Rapid Identification of *Mycobacterium avium subsp. avium* from MB/BacT Bottles Using PCR

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Abstract

The mycobacterial infections caused by strains of *Mycobacterium avium* complex (MAC) in animals and humans are still present. *Mycobacterium avium subsp. avium* (MAA) is the etiologic agent of avian tuberculosis, a chronic contagious disease described in a wide variety of domestic and wild bird species. The aim of this study was to evaluate the advantage of using the MB/BacT bottles in an automated system and PCR method for rapid identification of the etiologic agent of tuberculosis in hens. The culture samples isolates from hens in the MB/BacT automated system and PCR amplification of insertion element (IS1245, IS901) it was a faster and specific method.

Keywords: *IS1245, IS901, MB/BacT, Mycobacterium avium subsp. avium*

INTRODUCTION

The importance of mycobacterial infections caused by strains of *Mycobacterium avium* complex (MAC) in animals and humans is continuously increasing (Falkinham, 1996; Inderlied *et al.*, 1993; Pavlik *et al.*, 2000).

Mycobacterium avium species consists of four subspecies: *M. avium subsp. avium* (MAA), *M. avium subsp. hominissuis* (MAH), *M. avium subsp. silvaticum* (MAS), and *M. avium subsp. paratuberculosis* (MAP) (Mijs *et al.*, 2002; OIE, 2014; Thorel *et al.*, 1990), in turn classified into the S (sheep) and C (cattle) types. These subspecies, although closely related are different, each with specific pathogenetic and host range characteristics, ranging from environmental opportunistic bacteria that cause infections in swine and immunocompromised patients to pathogens of birds and ruminants (Laura Rindi and Grazelli, 2014; Thorel *et al.*, 1990).

Avian tuberculosis is one of the most important diseases that affects most of the birds (Mansour *et al.*, 2013; OIE, 2014; Tell *et al.*, 2001; Van Dar Heyden *et al.*, 1997). Avian tuberculosis is the most frequently produced by infection with *Mycobacterium avium subsp. avium* (serotypes 1, 2 and 3, containing specific gene segment *IS901* and nonspecific segment *IS1245*) and less frequently by *M. genavense* (Dvorska *et al.*, 2007; Fulton and Sanchez, 2008; Guerrero *et al.*, 1995; Mansour *et al.*, 2013; OIE, 2014; Pavlik *et al.*, 2000; Tell *et al.*, 2001). Avian tuberculosis is caused by other two members of *M. avium* complex: *M. avium subsp. hominissuis* (serotypes 4–6, 8–11 and 2; lacking gene segment IS901 and containing segment IS1245) and *M. intracellulare* (serotypes 7, 12–20 and 22–28; lacking both gene segments IS901 and IS1245) and by *M. intracellulare*, *M. scrofulaceum*, *M. fortuitum* and other potentially pathogenic mycobacterial species (OIE, 2014).

MAA is characterised by the presence of 2 to 17 copies of the *IS901* insertion sequence (Dvorska *et al.*, 2003; Inglis *et al.*, 2003; Kaevska *et al.*, 2010) and a single copy of *IS1245* (Johansen *et al.*, 2007). IS901 has only been detected in *M. avium* strains with serotypes 1, 2 and 3 (Pavlik *et*

al., 2000; Ritacco *et al.*, 1998) which is considered more pathogenic to birds than other serotypes (OIE, 2014; Tell *et al.*, 2001).

MAA remains the most prevalent agent of avian tuberculosis in the domestic hens (Gonzalez *et al.*, 2002; Kaevska *et al.*, 2010; Shitaye *et al.*, 2008). MAA was isolated as non-tuberculous mycobacteria (NTM) species from opportunistic infections in humans (Kaevska *et al.*, 2010; Pavlik *et al.*, 2000).

It is considered that under favourable conditions, all species of birds are susceptible to MAA infection, although among domestic birds, hens (*Gallus domesticus*) are the most susceptible species (Shitaye *et al.*, 2008).

One of the most important ways to reduce the incidence of infection with mycobacteria is by using a rapid diagnosis, particularly for high numbers of bacilli in sample who pose a greater risk of transmission (Gil-Setas *et al.*, 2004).

Considering that the conventional culture methods such as the use of Lowenstein Jensen (LJ) medium which requires 3 to 6 weeks for its isolation, plus an additional 1 to 2 weeks for its identification and that we still face with mycobacterial infections is necessary a rapid detection (Naveen and Basavaraj, 2012).

According to the Centers for Disease Control (1995) recommendations for mycobacteriology laboratories, the use of liquid medium has become one of the main diagnostic techniques. However, many laboratories have not introduced radiometric methods because they require expensive specialised equipment, qualified personnel and the safe disposal of radioactive wastes (Gil-Setas *et al.*, 2004). The combined use of a liquid and a solid medium has been recommended (Gil-Setas *et al.*, 2004; Metchock *et al.*, 1999).

Considering these aspects, there is a need of a culture method that is reliable and which has a short turnaround time. All methods have their own advantages and disadvantages, starting from the LJ medium to the present and speedy automated methods like the MB/BACT device (Adler *et al.*, 2005; Naveen and Basavaraj, 2012). MB/BACT is a safer and rapid method because is an automatically method which contains liquid media and not requires any radioactive material (Naveen and Basavaraj, 2012). One of the disadvantages for culture in liquid medium is that it does not provide visible colonies (Mirovic and Lepsanovic, 2002).

This paper presents the results performed at the hans with clinical tuberculosis, that were diagnosticated in the Faculty of Veterinary Medicine in Iasi.

For the detection of the subspecies *Mycobacterium avium subsp. avium* in tissues of hens it was used an MB/BacT automated system and PCR assay with the purpose to facilitated a rapid decision regarding the tuberculosis outbreak.

MATERIALS AND METHODS

A total of nine birds from species *Gallus domesticus* were examined. All the hens were originated from different households in Iași county.

The health status of the hens was evaluated for clinical signs of diarrhoea, emaciation and weakness that have evolved with a sporadic death. During the dissection, the organs were examined for the presence of tuberculous lesions.

Gross examination and histopathology. The tissue samples from naturally dead hens were rapidly examined for the presence of tuberculous lesions. The organs which had tuberculous lesions (liver, spleen and intestine), were formalin fixed, embedded in paraffin blocks and stained by Ziehl-Neelsen (ZN) technique for the presence of acid-fast bacilli (AFB) and hematoxylin eosin methylene blue (HEA) for histological evaluation of the granulomatous lesions. Histological samples were examined with a microscope Leica ICC50 HD using a 1000 magnification under oil immersion and capturing images with the Acquire Leica Software system.

Isolation of mycobacteria. The smears prepared from samples with typical lesions were stained according to the ZN technique for the presence of acid-fast bacilli (AFB).

For culture examination, the hens samples were concentrated and decontaminated using the modified Petroff`s technique with 4% sodium hydroxide (Allen and Baker, 1968).

The inoculum was digested and planted into three tubes slants of LJ medium and Herrolds‘s egg yolk media (HEYM) with Mycobactin J for each specimen. For shorter incubation times was used cultivation on MB/BacT bottles (bioMérieux, France), incubated at automated MB/BacT system (Organon Teknika). The MB/BacT bottles were analyzed every 10 minutes using the standard BacT/ALERT software. Incubation was performed

at 37°C for two months. The positive culture tubes were examined using ZN staining to observe a possible contamination and to confirm the presence of AFB.

Identification of isolates. The detection method was based on a specific and sensitive PCR for insertion element which enabled identification of MAA.

DNA was extracted from single colonies and was prepared for mycobacteria identification by performing the heat shook.

Mycobacterium avium subsp. avium CECT 7407 was used as the reference strain. All positive isolates, both in the solid and liquid medium, were examined by the PCR method for detection of IS1245, IS901 a specific insertion sequences for *M. avium* subspecies, using primers (Tab. 1) according to Miller *et al.*, (1999).

The PCR assay was performed using the kit IQ™ Supermix (BIO-RAD). IQ™ Supermix contains the following reagents dNTPs, iTaq DNA polymerase, 6 mM $MgCl_2$ and stabilizers. For IS901 and IS1245 PCR were used 5 µl of DNA template and 45 µl PCR mix: 15 µl water; 20 µl IQ supermix 1x, 5 µl forward and reverse primer 20 pmol.

The amplification conditions for primers IS901 and IS 1245 were as follows: 10 min at 94°C; 30 cycles of 60 s at 94°C, primer annealing at 62°C for 30 s, elongation 1 min at 72°C using a thermocycler (model MJ Mini Thermal Cycler, BIO-RAD), with a 10 min final extension at 72°C. Amplification was analyzed by electrophoresis with 5µl sample in 1% agarose and staining with ethidium bromide.

RESULTS AND DISCUSSIONS

Clinic signs and pathological lesions. In most cases, the infected birds showed no clinical signs, but they were lethargic and emaciated. At four hens it was observed a deformation of the breast bone. Affected birds were older than one year.

At post-mortem examination was observed severe lesions in the intestinal tract with typical caseous lesions in the liver and spleen. Most often these lesions were present in the liver, less frequently in the spleen and sporadically in the small intestine. The liver and spleen were

Tab. 1. Oligonucleotide primers used for PCR amplification of mycobacterial DNA

Organism(s)	Insertion sequence	Amplicon size (bp)	Sequence (5'-3')	Source
M. avium complex	IS1245	427	GCCGCCGAAACGATCTAC AGGTGGCGTCGAGGAAGA	Miller *et al.*
M. avium subsp. avium	IS901	252	GCAACGGTTGTTGCTGAAA TGATACGGCCGGAATCGCGT	Miller *et al.*

Fig. 1. Hens. Nodular granulomatous lesions in the liver

hypertrophied, exceeding the normal size, with increased friability and with the presence of granulomatous nodules of considerable size (Fig.1).

Gross examination and histopathology. The histopathological lesions in the liver, spleen and intestine were characteristic with infection of mycobacteria (avian tuberculosis), with presence of the granulomas in various stages of development and with the observation of three types of nodules.

It was found both the granuloma which contain necrosis (Fig. 2) as well as the non-necrotic granuloma.

The formation of the granuloma was characterized by the presence of multinucleated giant cells with nuclei arranged like a horseshoe (Langhans giant cell) and for the mature granuloma it was observed a caseous central necrosis (Fig. 2). The central caseum was surrounded by epithelioid macrophages, Langhans-type giant cells, peripheral lymphocytes and collagen fibres (fibrosis).

In conclusion, all hens showed lesions with typical granulomas in liver, spleen and intestine, with central necrosis and cellular components of chronic inflammation and with detection of numerous acid-fast bacilli.

The diagnosis of *M. avium* infection is based on clinical signs, postmortem gross lesions, and by observing the acid-fast bacilli in crushed lesions using microscopy, which is sufficient for a positive diagnosis (Fulton and Thoen, 2003; Tell *et al*, 2001; Kuldeep *et al.*, 2011).

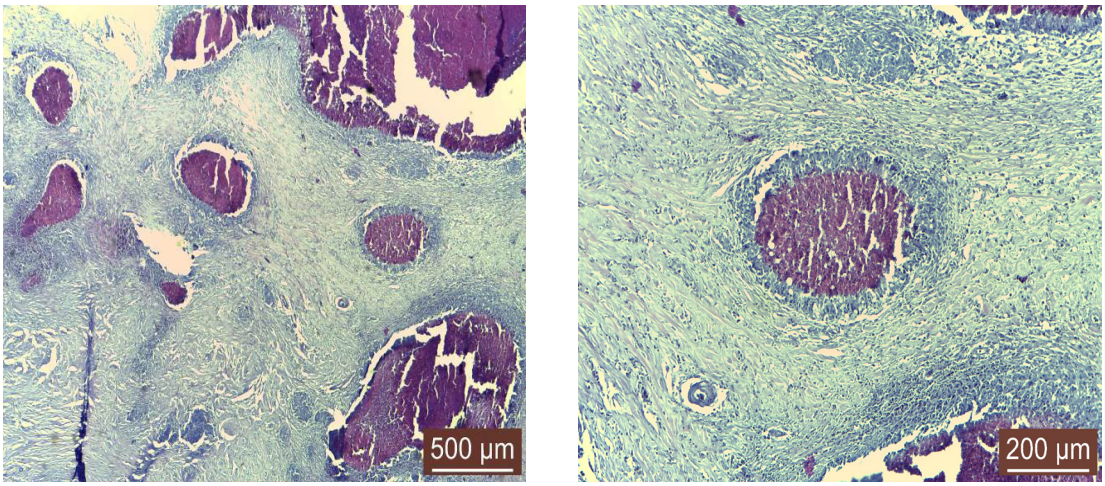
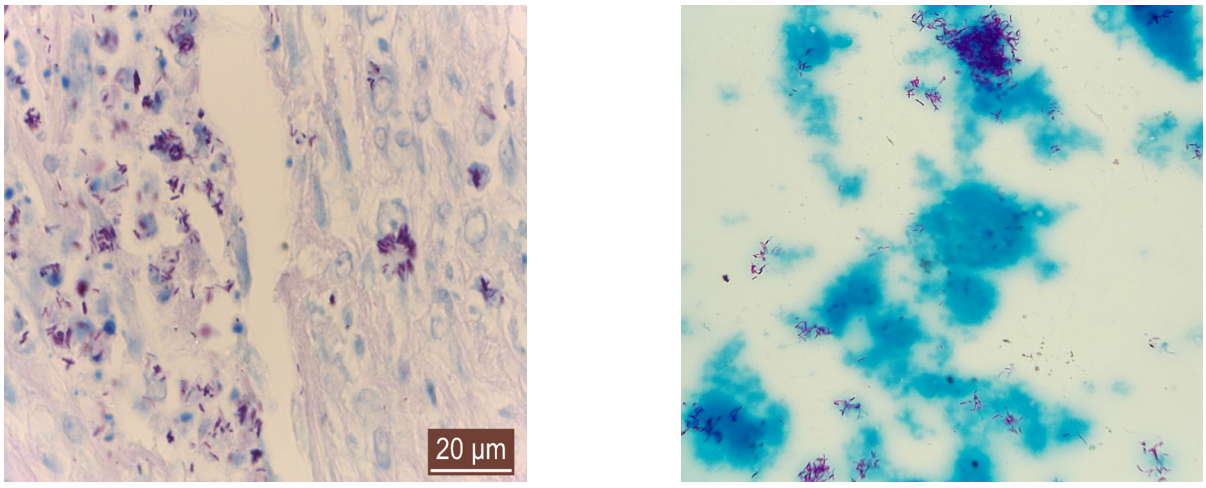


Fig. 2. Hens. Liver. Tuberculous granulomas; HEA



A B

Fig. 3. Hens. Spleen. A. – AFB in tissues; B. – AFB in the obtained culture on MB/BacT, ZN

Culture examination. After culture examination, there obtained isolates from all hens. After incubation there produced typical smooth colonies transparent in both medium. The growth from the cultures were confirmed by ZN staining proving infection with mycobacteria (Fig. 3).

In this study was attempted the feasibility of using MB/BacT and LJ as the primary isolation media for mycobacteria. The two media were compared regarding the number of isolates, the rate of isolation and the mean duration of the isolation. In addition, the isolates from both MB/BacT and LJ medium was verified by PCR.

At a hen the sample on the LJ medium and HEYM with mycobactin, could not be isolated, but in MB/Bact it was observed a positive sample (Tab. 2).

Tab. 2. Comparison of MB/BacT and LJ for duration of isolation and number of isolates of MAA

No of sample	Duration of isolation (days)		Culture positive	
	LJ	MB/BacT	LJ	MB/BacT
1	6	4	+	+
2	15	8	+	+
3	9	6	+	+
4	12	8	+	+
5	20	9	-	+
6	14	8	+	+
7	10	5	+	+
8	11	6	+	+
9	11	5	+	+
Mean duration / No of isolates	12	6.5	8/9	9/9

The mean duration of the isolation on LJ and MB/BacT was 12 days and 6.5 days respectively (Tab.2). The difference between the LJ medium and the MB/BacT medium was significant. The liquid media in MB/BacT proved to be more successful comparative with LJ, because is faster (6.5 days compared to 12 days).

MB/BacT it proved to be superior to LJ medium in the isolation rate, because it could isolate mycobacteria with 7-10 days earlier (Naveen and Basavaraj, 2012).

PCR identification. All isolated strains obtained at hens on LJ and MB/BacT medium, were detected by PCR.

The isolates were positive for IS901 and IS1245 PCR, with the visualization of a 252 bp fragment and 427 bp fragment, respectively.

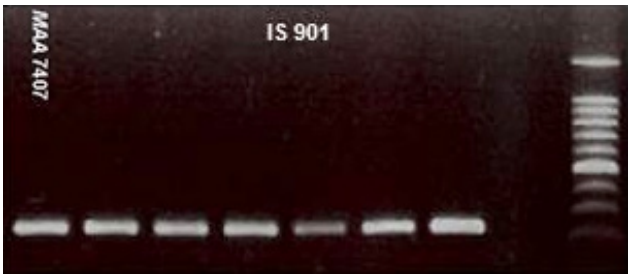
After performing the PCR, all hens (n=9) were found positive for both IS1245 and IS901 (Fig. 4), confirming infection with *Mycobacterium avium subsp. avium*.

According to the results obtained at PCR we can reach to the conclusion that MB/Bact automated system is a faster and also reliable alternative to conventional culture methods.

CONCLUSION

Diagnosis of MAA (containing IS901) from tissue samples was confirmed using conventional PCR.

The sensitivity and the time to detection were significantly better with MB/BacT than with solid LJ medium. The culture samples isolated from hens in the MB/BacT automated system and PCR amplification of insertion element were rapid and specific methods.



A

B

Fig.4. –PCR amplification product of the 252 bp specific fragment from IS901;
IS 901 PCR performed on extract isolated from MB/BacT;
A – samples for 6 hens; B – samples for 3 hens; MAA 7407 – postive control.

The results indicates that the MB/BacT system has great potential: it is a rapid, sensitive, safe, simple-to-use, and an automated method for the isolation of mycobacteria.

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